Isolation, structure and synthesis of chevalierins A, B and C, cyclic peptides from the latex of *Jatropha chevalieri*

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From the EtOAc extract of the latex of *Jatropha chevalieri* (Euphorbiaceae), two cyclooctapeptides, chevalierins A (1) and B (2), and a cyclononapeptide, chevalierin C (3), were isolated by a multi-step chromatography procedure, including HPLC. Their structures were elucidated by chemical degradation, mass spectrometry, homonuclear and heteronuclear NMR experiments and confirmed from their synthesis. Their antimalarial activities were examined.

Introduction

Latices of *Jatropha* species (Euphorbiaceae) have been shown to be a rich source of bioactive cyclic peptides. At first, labaditin,[1] a cyclic decapeptide and biobollein,[2] a cyclic nonapeptide were isolated from the latex of *J. multifida* on the basis of immunomodulatory activity-guided purification and both peptides selectively inhibited the classical pathway of human complement activation. The cyclic octapeptide curcacycline A,[3] with a similar immunosuppressive activity, was isolated from the latex of *J. curcas* along with curcacycline B, a cyclic nonapeptide which enhanced rotamase activity of cyclophilin B.[4] Podacyclines A and B,[5] nona- and heptacyclopeptides respectively, were characterized in the latex of *J. podagrica* and cyclogossines A[6] and B,[7] hepta- and octacyclopeptides in *J. gossypifolia*. This led us to investigate other *Jatropha* species for the presence of bioactive peptides.

The latex of *J. chevalieri* Beille, a small shrub in Senegal, is used locally to treat infected wounds in folk medicine.[8] Investigation on constituents of this latex resulted in isolation of three new cyclic peptides named chevalierin A (**1**), B (**2**) and C (**3**). As it is known that some natural cyclopeptides such as apicidins[9] or cyclosporins[10] have potent antiparasitic effects against *Plasmodium*, we examined the *Plasmodium falciparum* antiproliferative activity of these cyclopeptides. Only **1** showed a moderate antimalarial activity (IC_{50} = 8.9 μM).

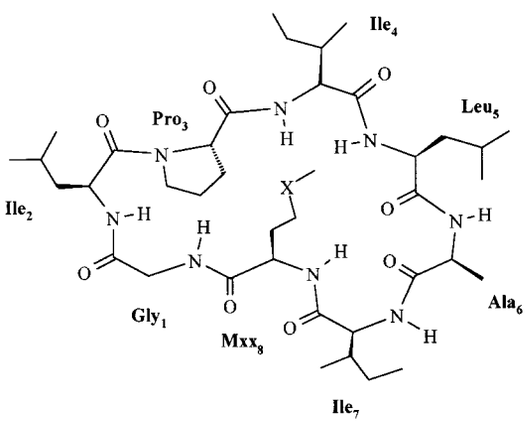
This paper deals with the isolation, structure elucidation and synthesis of compounds **1**–**3**.

Results and discussion

Isolation and characterization of 1, 2 and 3

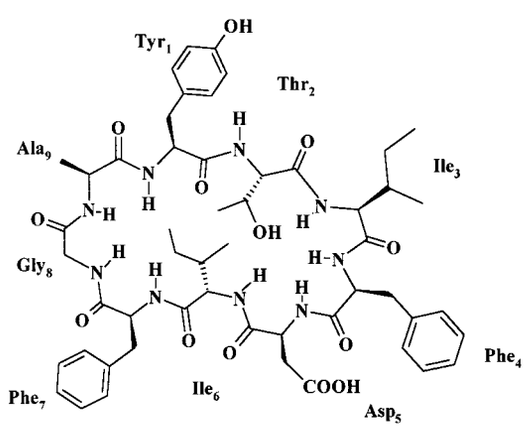
The latex of *J. chevalieri* was partitioned between ethyl acetate and water. The organic phase soluble material was fractionated by exclusion chromatography to yield two peptide fractions. Each of them was analysed by C_{18} reversed-phase HPLC. The first group revealed two peptide components named chevalierins A (**1**) and B (**2**) (Fig. 1a) and the second one, only one peptide named chevalierin C (**3**) (Fig. 1b). Each peptide was purified by repetitive semi-preparative HPLC and proved to be homogeneous by further LSIMS and NMR analyses. The three compounds gave a positive reaction with the chlorine–*o*-tolidine reagent suggesting the presence of amide groups and a negative reaction with ninhydrin, indicating the absence of a free amino group and suggesting a cyclic peptide structure.

The amino acid composition was determined by HPLC analysis of the complete acid hydrolysates of the peptides and was characterised by the presence of 1 Ala, 1 Gly, 3 Ile, 1 Leu, and 1 Pro for both **1** and **2**. The distinction between **1** and **2** arose from the presence of one methionine in **1** and one methionine sulfoxide in **2**. The amino acid composition of **3** similarly determined was: Ala (1), Asp (1), Gly (1), Ile (2), Phe (2), Thr (1) and Tyr (1). The absolute configuration of the chiral amino acids was shown to be L from the derivatization of the acid hydrolysates to *N*-trifluoroacetyl isopropyl esters followed by GC analysis on a chiral capillary column.



1 Mxx_8 = Met (X : S)

2 Mxx_8 = Mso (X : S=O)



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Sequence determination of chevalierin A

The molecular weight of 808 for **1** was deduced from the positive

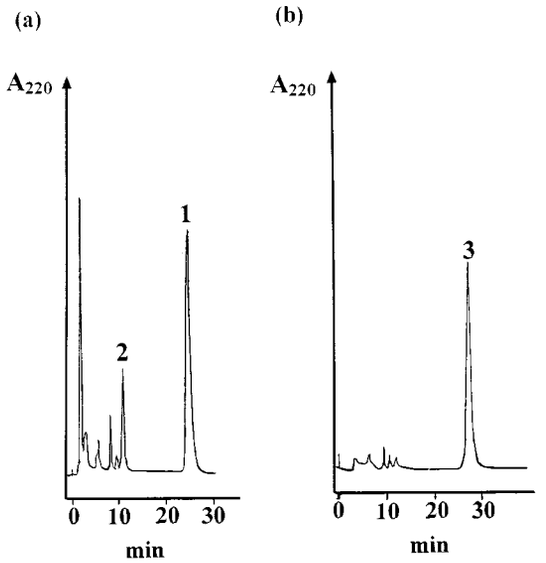


Fig. 1 HPLC chromatograms of natural (a) **1** and **2**, and (b) **3**. Conditions: C_{18} reversed-phase Kromasil, 5 μm (250 × 7.8 mm); flow rate 2 cm^3 min^{-1}; mobile phase MeOH–H_2O 77 : 23; det. UV 220 nm.

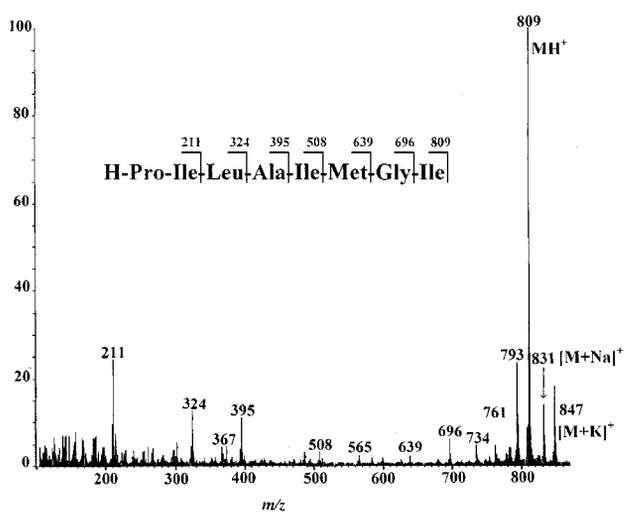


Fig. 2 Positive LSIMS of chevalierin A.

LSIMS spectra which displayed the protonated molecule MH^+ (base peak) at *m/z* 809 and the adduct ions $[M + Na]^+$ and $[M + K]^+$ at *m/z* 831 and 847, respectively (Fig. 2). Compound **1** showed a high-resolution LSIMS protonated molecule MH^+ at *m/z* 809.4951 corresponding to the molecular formula $C_{39}H_{69}N_8O_8S$ (calc. 809.4959). The molecular weight of **1** fits well with the above amino acid composition in a cyclic octapeptide.

The NMR sequence determination of **1** was obtained by assignments of different spin systems to residue types from ^1H–^1H COSY, HOHAHA and ROESY experiments, and sequential assignments were further afforded by exploitation of *d*NN (*i*, *i* + 1) and d*αN* (*i*, *i* + 1) ROESY connectivities and confirmed by long range ^1H–^{13}C HMBC experiments. Complete assignments of ^1H and ^{13}C NMR signals were obtained in a polar solvent such as DMSO-d_6. Though **1** contains one proline residue, the 1D NMR spectra of **1** gave well resolved sharp signals and the presence of minor conformers was not observed. The ^1H NMR spectrum showed a doublet and a singlet methyl signals at δ 1.32 and 2.02 ppm ascribable to Ala and Met, respectively. Complete assignment of ^1H chemical shifts to specific protons of individual residues was obtained by 2D homonuclear COSY and TOCSY experiments (Fig. 3a, b) showing complete spin systems of 1 Ala, 1 Gly, 3 Ile, 1 Leu, 1 Met and 1

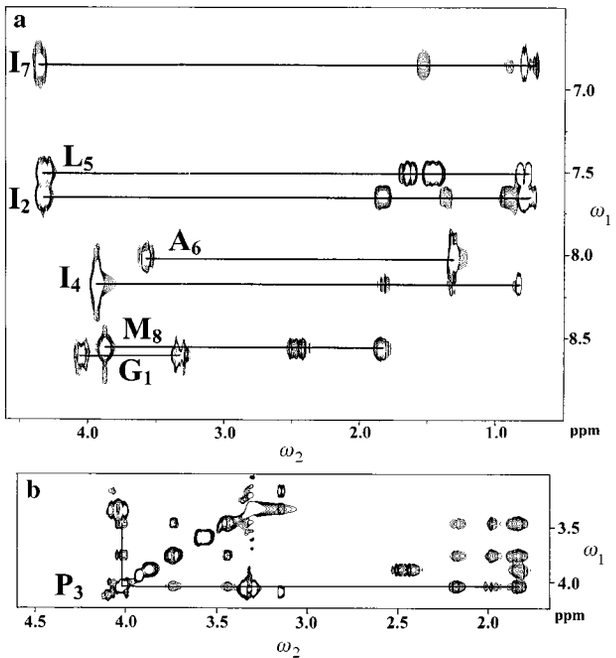


Fig. 3 Expansion of the TOCSY spectrum of **1** in DMSO-d_6: **a**: ω_2 = 0.6–4.5 ppm, ω_1 = 6.6–8.9 ppm; **b**: ω_2 = 1.6–4.5 ppm, ω_1 = 3.3–4.2 ppm; spin-systems are labelled with the sequential residue positions.

Pro. The corresponding carbon resonances were determined on the basis of *J*-modulated ^{13}C, HMQC and HMBC experiments (Table 1). The connectivities between neighbouring amino acids were determined from the ROESY spectrum and were completely carried out using inter-residue *d*αN (*i*, *i* + 1) and *d*NN (*i*, *i* + 1) connectivities. The lowest field NH proton signal of glycine at 8.59 ppm was assigned to Gly_1. Inter-residue *d*αN (*i*, *i* + 1) connectivities were found between each adjacent residue extending from Gly_1 to Ile_2 and from Pro_3 to Gly_1, as well as the ROE connectivities between Ile_2-Hα and Pro_3-Hδ. In addition, *d*NN (*i*, *i* + 1) connectivities were observed between Gly_1 and Ile_2 and from Ile_4 to Ile_7 (Fig. 4). Accordingly, the structure of chevalierin A was determined as cyclo(-Gly_1-Ile_2-Pro_3-Ile_4-Leu_5-Ala_6-Ile_7-Met_8-). Chemical shifts of β and γ carbons of Pro were 28.9 and 24.5 ppm, respectively giving evidence for the presence of a *trans*-proline amide bond;[11] strong ROE enhancements between the α proton of Ile_2 and δ protons of Pro_3 added evidence of the *trans* geometry.

The LSIMS spectrum depicted slight, but significant ions supporting the amino acid sequence derived from the NMR experiments. The data were consistent with the initial cleavage of the ring at the Ile_2–Pro_3 amide bond, to give a linear N-protonated acylium ion corresponding to the octapeptide which further sequentially lost the following residues (*m/z*): Ile (696), Gly (639), Met (508), Ile (395), Ala (324) and Leu leading to the N-protonated dipeptide Pro_3-Ile_4 acylium ion at *m/z* 211 (Fig. 2).

Sequence determination of chevalierin B

Chevalierin B showed the protonated molecule MH^+ at *m/z* 825 and the adduct ions $[M + Na]^+$ (base peak) and $[M + K]^+$ at *m/z* 847 and 863 in the LSIMS spectrum. The molecular formula $C_{39}H_{69}N_8O_9S$ of **2** was established by HR-LSIMS [*m/z* 825.4911 (MH^+), calc. 825.4908]. The 16 mass units difference between **1** and **2**, and the assignment to an additional oxygen atom suggested the presence of a methionine sulfoxide (Mso) instead of a Met as in **1**. This result was confirmed by the LSIMS spectrum in which a series of protonated acylium ions identical to those of **1** were found at *m/z* 211, 324, 395 and 508 corresponding to Pro_3-Ile_4, Pro_3-Ile_4-Leu_5, Pro_3-Ile_4-Leu_5-Ala_6 and Pro_3-Ile_4-Leu_5-Ala_6-Ile_7 fragments, respectively. The mass

Table 1 ^{1}H and ^{13}C NMR spectral data for **1** and **2** (DMSO-d_6, 298 K).

		1		**2**	
Residue		δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C
Gly$_1$	NH	8.59		8.69	
	α	4.05	42.6	4.07 (dd, 16.5, 8.1)	42.6
	α′	3.35		3.35	
	CO		168.9		168.9
Ile$_2$	NH	7.64 (d, 9.1)		7.67 (d, 9.1)	
	α	4.30	55.2	4.30	55.2
	β	1.83	35.3	1.81	35.3
	γ_1	1.37	23.5	1.35	23.5
	$CH_3(\gamma_2)$	0.90	16.6	0.87	16.6
	$CH_3(\delta)$	0.78	11.8	0.81	11.8
	CO		170.6		171.1
Pro$_3$	α	4.00	62.8	4.04	62.8
	β	2.18	28.9	2.22	28.9
	β′	1.85		1.85	
	γ	1.98	24.5	1.95	24.5
	γ′	1.85		1.85	
	δ	3.72	47.1	3.78	47.0
	δ′	3.43		3.42	
	CO		174.7		174.8
Ile$_4$	NH	8.17 (d, 4.5)[a]		8.22 (d, 3.6)	
	α	3.90	60.0	3.94	60.0
	β	1.83	35.0	1.85	35.0
	γ_1	1.35	25.6	1.49	25.7
	$CH_3(\gamma_2)$	0.84	15.4	0.85	15.4
	$CH_3(\delta)$	0.76	11.3	0.76	11.3
	CO		170.2		170.5
Leu$_5$	NH	7.50 (d, 9.4)		7.51 (d, 9.4)	
	α	4.30	50.2	4.26	50.2
	β	1.65	40.0	1.69	40.0
	β′	1.45		1.49	
	γ	1.47	24.1	1.49	24.1
	$CH_3(\delta_1)$	0.77	20.2	0.82	20.2
	$CH_3(\delta_2)$	0.82	23.2	0.78	23.2
	CO		171.1		171.3
Ala$_6$	NH	8.01 (d, 6.9)		7.99 (d, 7.0)	
	α	3.55	50.0	3.57	49.8
	β	1.32 (d, 7.0)	15.0	1.32 (d, 6.9)	14.9
	CO		170.2		170.1
Ile$_7$	NH	6.85 (d, 8.9)[a]		6.79 (d, 8.2)	
	α	4.35	55.2	4.39	55.4
	β	1.50	38.5	1.53	39.0
	γ_1	1.35	23.5	1.32	23.4
	$CH_3(\gamma_2)$	0.80	14.8	0.83	14.7
	$CH_3(\delta)$	0.70	11.3	0.72	11.3
	CO		172.4		172.4
Mxx$_8$	NH	8.58		8.73	
	α	3.87	54.2	3.88	54.2
	β	1.83	29.5	1.92	22.9
					23.0
	γ	2.45	29.4	2.77 and 2.65	48.9
					49.1
	$CH_3(\delta)$	2.02 (s)	14.4	2.52 and 2.53	37.9
	CO		171.6		171.4

[a] Determined at 313 K; Mxx = Met for **1** or Mso for **2**.

spectrum of **2** exhibited two more peaks at *m*/*z* 318 and 431 which could be assigned to the protonated Ile$_7$-Mso$_8$-Gly$_1$ and Ile$_7$-Mso$_8$-Gly$_1$-Ile$_2$ ion fragments. The ^{1}H and ^{13}C chemical shifts of each amino acid of **2** were assigned from the detailed analyses of the 2D NMR data (COSY, HOHAHA, HMQC and HMBC experiments). The ^{1}H NMR signals due to the γ-methylene and the δ methyl groups in Mso were observed as a pair of signals in the ratio 1 : 1 at δ 2.65 and 2.77 for $CH_2\gamma$ and at δ 2.52 and 2.53 for $CH_3\delta$ implying that the sulfoxide group of the Mso residue was racemic.[12–14] In addition, the ^{13}C NMR peaks ascribable to β, γ and δ signals of Mso were also split: 22.9 and 23.0 ppm for $CH_2\beta$, 48.9 and 49.1 ppm for $CH_2\gamma$ and 37.87 and 37.91 ppm for $CH_3\delta$ (Table 1).

Sequence determination of chevalierin C

The LSIMS spectrum of **3** had the protonated molecule MH^+ at *m*/*z* 1028 as the base peak and no adduct ions were observed. Its molecular formula was determined as $C_{52}H_{69}N_9O_{13}$ by HR-LSIMS (MH^+ *m*/*z* 1028.5120, calc. 1028.5093) in agreement with the amino acid composition and a cyclopeptide structure.

The ^{1}H NMR spectra in DMSO-d_6 at 298 K gave large and weakly resolved signals, probably indicating a conformational variability. An increase in the temperature allowed a resolution improvement and finally all the ^{1}H and ^{13}C NMR experiments were recorded at 328 K and gave well-resolved sharp signals. The presence of minor conformers was not detected at this temperature. All proton resonances of **3** were assigned by analysis of COSY and TOCSY spectra (Table 2). One-bond ^{1}H–^{13}C correlation maps (HMQC) and two or three-bond ^{1}H–^{13}C heteronuclear couplings (HMBC) allowed the assignment of the ^{13}C signals including those of the quaternary

Table 2 ^{1}H and ^{13}C NMR spectral data for **3** (DMSO-d_6, 328 K).

Residue		δ_H (int., mult., *J* in Hz)	δ_C	Residue		δ_H (int., mult., *J* in Hz)	δ_C
Tyr_1	NH	8.12 (d, 7.7)		**Asp_5**	NH	7.76	
	α	4.12	55.9		α	4.50	50.2
	β	3.00	34.4		β	2.73 (dd, 7.0, 15.2)	36.7
	β′	2.97			β′	2.57 (dd, 6.5, 15.2)	
	OH	9.02 (br s)			CO (γ)		172.6
	γ		128.2		CO		171.8
	δ	6.98	129.6				
	ε	6.64	114.8	**Ile_6**	NH	7.93 (d, 5.7)	
	ζ		155.5		α	3.80	5.92
	CO		170.8		β	1.57	35.5
					γ_1	1.13	24.3
Thr_2	NH	7.52 (d, 8.9)			γ_1'	0.98	
	α	4.47	57.1		$CH_3(\gamma_2)$	0.70	14.7
	β	4.20	67.2		$CH_3(\delta)$	0.55 (d, 6.8)	11.0
	γ	1.06 (d, 6.3)	19.0		CO		170.5
	CO		170.5				
				Phe_7	NH	7.99 (d, 8.0)	
Ile_3	NH	7.80 (d, 8.4)			α	4.50	53.8
	α	3.90	58.3		β	3.20	35.9
	β	1.63	35.3		β′	2.82	
	γ_1	1.13	23.8		γ		138.0[a]
	γ_1'	0.95			δ	7.20	128.6[b]
	$CH_3(\gamma_2)$	0.70	14.9		ε	7.20	127.8[c]
	$CH_3(\delta)$	0.67	11.0		ζ	7.17	125.9
	CO		170.9		CO		170.9
Phe_4	NH	8.07 (d, 6.7)		**Gly_8**	NH	7.60 (br s)	
	α	4.28	54.8		α	3.87	41.7
	β	2.82	36.3		α′	3.55 (dd, 4.9, 16.5)	
	β′	3.18			CO		169.0
	γ		137.9[a]				
	δ	7.20	128.8[b]	**Ala_9**	NH	7.85 (d, 9.0)	
	ε	7.20	127.9[c]		α	4.10	48.7
	ζ	7.17	125.9		β	1.13 (d, 7.1)	17.1
	CO		170.0		CO		172.2

[a,b,c] May be reversed.

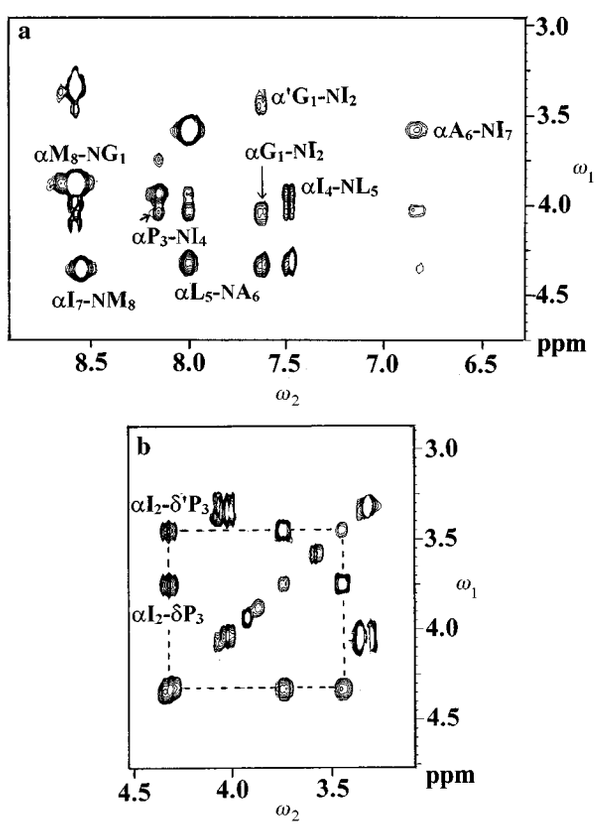


Fig. 4 Parts of the ROESY spectrum of **1** in DMSO-d_6: **a**: ω_2 = 6.4–8.8 ppm, ω_1 = 3.0–4.7 ppm; **b**: ω_2 = 3.2–4.5 ppm, ω_1 = 3.0–4.7 ppm.

carbons (Table 2). Connectivities between neighbouring amino acids which were unequivocally established from the *dα*N (*i*, *i* + 1) ROESY cross peaks allowed the determination of the sequence as: cyclo(-Tyr_1-Thr_2-Ile_3-Phe_4-Asp_5-Ile_6-Phe_7-Gly_8-Ala_9-).

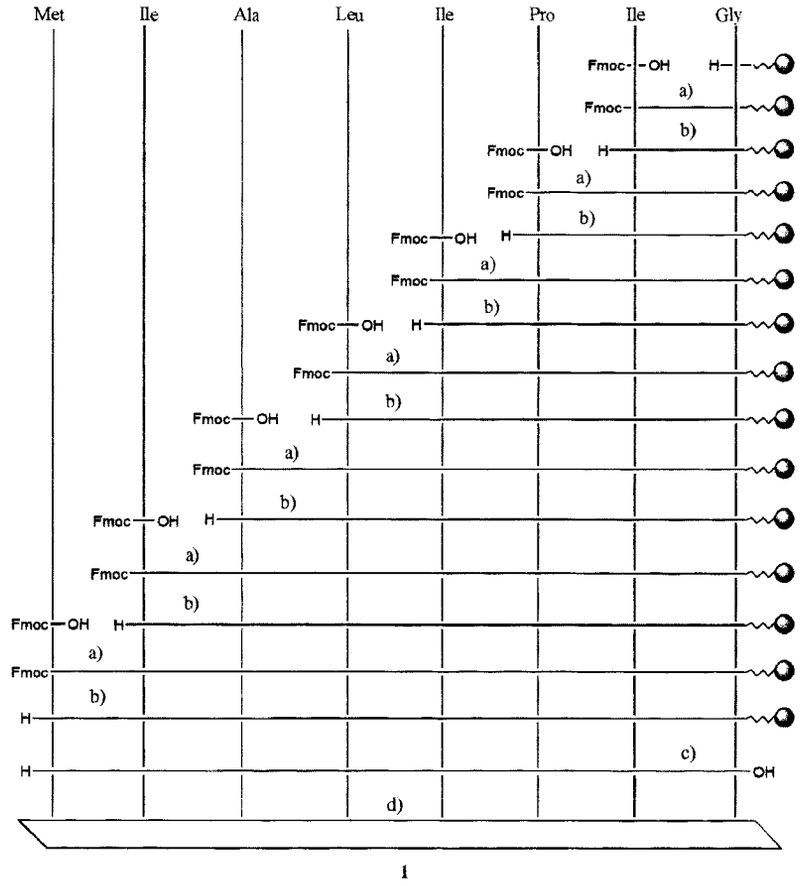
Synthesis of chevalierins A, B and C

The linear precursors of **1** and **3** were chosen with the glycine in C-terminal position to prevent racemization in the cyclization. These precursors were synthesized using solid-phase technique based on the 9-fluorenylmethoxycarbonyl (Fmoc) amino protection strategy employing chlorotrityl resin (cleavable with dilute TFA) avoiding diketopiperazine formation between the C-terminal glycine and the second amino acid (Ile or Ala) (Fig. 5 and 6). In the case of **3**, the *tert*-butyl (tBu) group was used as side chain protection for Tyr, Thr and Asp. After complete chain assembly, the linear peptide was cleaved from the solid support followed by precipitation of the crude material in diethyl ether. The purity was checked by reversed-phase HPLC. The structural integrity of linear precursors was assessed by electrospray mass spectrometry.

The cyclisation was accomplished in DMF under high dilution conditions (10^{-3} M) with 1.5 equivalents of hexafluorophosphate (HBTU) and 10 equivalents of triethylamine (NEt_3). The final cyclic peptide was extracted with AcOEt and purified to homogeneity by semi-preparative reversed-phase HPLC. Compounds **1** and **3** were obtained in 27 and 28% yields, respectively.

Compound **2** was obtained by oxidation of **1** with H_2O_2 (35% in H_2O) in DMSO at room temperature (oxidation yield: 80%).

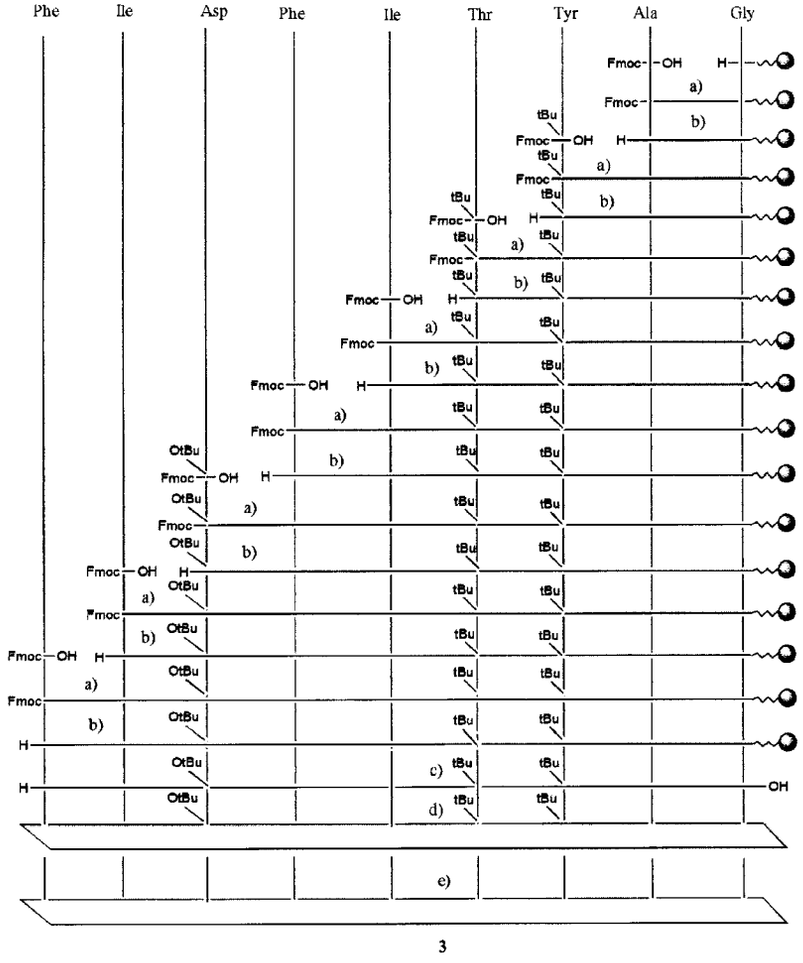
The three cyclopeptide structures were checked by mass spectrometry and NMR experiments and by spectral data comparison with the natural peptides.



1

a) HBTU/HOBt/DMF
b) Piperidine 20% in DMF
c) TFA 0.5% in DCM
d) HBTU/DMF 10^{-3}M

Fig. 5 Total synthesis of **1**.



3

a) HBTU/HOBt/DMF
b) Piperidine 20% in DMF
c) TFA 0.5% in DCM
d) HBTU/DMF 10^{-3}M
e) TFA

Fig. 6 Total synthesis of **3**.

Experimental

Plant material

Jatropha chevalieri Beille growing in the region of Dakar (Senegal) was collected in September 1996, identified and a voucher specimen was deposited in the National herbarium, Laboratoire de Phanérogamie, Muséum National d'Histoire Naturelle, Paris. Crude latex was obtained by cutting off leaf stalks and adding a few drops of EtOH to prevent the latex from excessive foaming, and was stored at −20 °C until use.

Extraction and isolation

To 60 ml of crude latex, 40 ml of demineralized H_2O were added and the mixture extracted with 3 × 100 ml of EtOAc. The solvent was removed by evaporation under reduced pressure and the crude residue (390 mg) dissolved in MeOH and chromatographed on Sephadex LH-20 (Pharmacia) with MeOH as eluent. Two peptide-containing fractions were monitored by TLC (SiO_2, Merck 60 F_{254}) with CH_2Cl_2–MeOH 85:15 as eluent system. Peptides were detected with the Cl_2–*o*-tolidine reagent. The first peptide group (80 mg) exhibited two blue spots: R_f 0.71 (**1**); R_f 0.24 (**2**); the second one (42 mg) revealed one spot of R_f: 0.40 (**3**). The two peptide groups were purified by reversed-phase HPLC (Kromasil C_{18}, 250 × 7.8 mm, 5 μm, AIT France; 23% H_2O in MeOH; flow rate 2 cm^3 min^{-1}; detection 220 nm) to yield chevalierin A (**1**) (t_R: 26 min, 57.3 mg), chevalierin B (**2**) (t_R: 12 min, 14.2 mg) and chevalierin C (**3**) (t_R: 28 min, 13.4 mg).

General data

The $[a]_D$ values were determined with a Perkin-Elmer Model 243 B polarimeter.

1: $[a]_D^{22}$ −13 (*c* 0.18, MeOH)
2: $[a]_D^{22}$ −11 (*c* 0.33, MeOH)
3: $[a]_D^{22}$ −114 (*c* 0.33, MeOH)

Amino acid composition and absolute configuration

Chevalierins A, B and C (1 mg) were hydrolyzed with 6 M HCl (0.5 ml) in a sealed tube at 110 °C under argon for 24 h. After hydrolysis, the reagents were removed under reduced pressure. The residues were dissolved in 0.2 M Na citrate buffer (pH 2.2) and amino acid composition was determined by cation-exchange chromatography on a Liquimat 2 Amino Acid Analyzer (Kontron) and detected with the OPA reagent. The absolute configuration of amino acids was determined on a chiral capillary column by CPG. The peptide hydrolysates obtained with 6 M HCl were dried over potassium hydroxide pellets. The crude residues were dissolved in an anhydrous solution of 3 M HCl in propan-2-ol and heated at 110 °C for 20 min. The reagents were evaporated under reduced pressure, the residues were dissolved in CH_2Cl_2 (0.5 ml), and 0.5 ml of trifluoroacetic anhydride was added. The mixtures were kept in a screw-capped tube at 100 °C for 5 min. The reagents were evaporated, and the GC analyses were performed on a Hewlett Packard series II 5890 gas chromatograph on a Chirasil-L-Val (*N*-propionyl-L-valine-*tert*-butylamide polysiloxane) quartz capillary column (Chrompack, 25 m length, 0.2 mm i.d.) with He (1.1 bar) as carrier gas and a temperature program of 50–130 °C at 3 °C min^{-1}, then 130–190 °C at 10 °C min^{-1}. The retention times of the *N*-trifluoroacetyl isopropyl ester derivatives were compared with those of commercial references.

Composition and absolute configuration of amino acids (number) **1**: L-Ala (1), Gly (1), L-Ile (3), L-Leu (1), L-Met (1) and L-Pro (1); **2**: Gly (1), L-Ile (3), L-Leu (1), L-Mso (1) and L-Pro (1); **3**: L-Ala (1), L-Asp (1), Gly (1), L-Ile (2), L-Phe (2), L-Thr (1) and L-Tyr (1).

Secondary ion mass spectrometry

Positive LSIMS spectra were recorded on a ZAB2-SEQ (VG Analytical, Manchester, UK) mass spectrometer equipped with a standard FAB source and a caesium ion gun operating at 35 kV. The peptide methanolic solution was mixed with α-monothioglycerol as matrix. Positive HR-LSIMS were recorded on a ZAB-HF spectrometer. The resolution was 2000.

Chevalierin A. *m*/*z* (relative intensity) 211 (24), 324 (15), 367 (5), 395 (13), 508 (5), 565 (4), 639 (3), 696 (6), 734 (5), 761 (5), 793 (23), 809 (MH^+, 100), 831 ($[M + Na]^+$, 14), 847 ($[M + K]^+$, 18).

Chevalierin B. *m*/*z* 211 (24), 318 (11), 324 (11), 395 (9), 431 (3), 508 (4), 727 (4), 783 (22), 799 (9), 809 (13), 825 (MH^+, 53), 847 ($[M + Na]^+$, 100), 863 ($[M + K]^+$, 33).

Chevalierin C. *m*/*z* 262 (5), 784 (2), 826 (6), 1028 (MH^+, 100), 1050 ($[M + Na]^+$, 23).

NMR Spectroscopy

^1H and ^{13}C NMR spectra were run in DMSO-d_6 using a Bruker AC 300 spectrometer, equipped with an Aspect 3000 computer using DISNMR software. A 10 mg sample of **1**, **2** and **3** in a 5 mm tube (0.5 ml of degassed DMSO-d_6) was used for homonuclear and heteronuclear measurements. The one dimensional ^1H NMR spectra (300.13 MHz, DMSO-d_6, 296 K) were obtained with 64–186 scans.

For **1**, the COSY spectrum was run with a total of 256 experiments of 40 scans each with a sweep width in F_2 of 2762 Hz (size 2K), zero filling in F_1 (size 1K). For the TOCSY spectra, 256 experiments of 128 scans were performed with a sweep width in F_2 of 3144 Hz (size 2K), zero filling in F_1 (size 1K); sine bell weighing functions shifted by π/2 in F_1 and F_2 were applied. A phase-sensitive ROESY NMR experiment was obtained with mixing time of 150 ms. A total of 256 experiments of 96 scans each were acquired with a sweep width in F_2 of 3144 Hz (size 2K), zero filling in F_1 (size 1K); sine bell weighing functions shifted by π/2 in F_1 and F_2 were applied.

For **2**, the COSY spectrum was run with a total of 256 experiments of 20 scans each with a sweep width in F_2 of 2857 Hz (size 2K), zero filling in F_1 (size 1K). For the TOCSY spectra, 256 experiments of 96 scans were performed with a sweep width in F_2 of 3144 Hz (size 2K) zero filling in F_1 (size 1K); sine bell weighing functions shifted by π/2 in F_1 and F_2 were applied. A phase-sensitive ROESY NMR experiment was obtained with mixing time of 150 ms. A total of 256 experiments of 96 scans each were acquired with a sweep width in F_2 of 3144 Hz (size 2K), zero filling in F_1 (size 1K); sine bell weighing functions shifted by π/2 in F_1 and F_2 were applied.

For **3**, all the NMR experiments were made at 328 K. The COSY spectrum was run with a total of 256 experiments of 16 scans each with a sweep width in F_2 of 2994 Hz (size 2K), zero filling in F_1 (size 1K). For the TOCSY spectra, 256 experiments of 96 scans were performed with a sweep width in F_2 of 3355 Hz (size 2K), zero filling in F_1 (size 1K); sine bell weighing functions shifted by π/2 in F_1 and F_2 were applied. A phase-sensitive ROESY NMR experiment was obtained with a mixing time of 150 ms. A total of 256 experiments of 96 scans each were acquired with a sweep width in F_2 of 3355 Hz (size 2K), zero filling in F_1 (size 1K); sine bell weighing functions shifted by π/2 in F_1 and F_2 were applied.

The ^{13}C NMR experiments were acquired at 75.47 MHz using a ^1H–^{13}C Dual probehead. The delay preceding the ^{13}C pulse for the creation of multiple quanta coherences through several bonds in the HMBC was set to 70 ms. The heteronuclear coupling constant value used in the HMQC experiment to establish the delay needed to select the protons coupled to carbon was 135 Hz.

Solid-phase peptide synthesis of 1 and 3

The linear precursors of **1** and **3** were prepared on a ABI433A Perkin-Elmer synthesizer using standard solid-phase synthesis techniques and the Fmoc–tBu protection strategy. Syntheses were performed starting with H-Gly-2-chlorotrityl-resin (Novabiochem, 0.9 mmol g^{-1}). Fmoc group removals were carried out

by treatment with 20% piperidine in DMF. Couplings were performed using 10 equivalents of Fmoc-amino acid with an equivalent amount of HBTU and HOBT (0.5 M) in DMF for 1–2 h. Cleavage and deprotection of the peptides were realized by shaking the complete protected peptidyl resin twice with 1% TFA in CH_2Cl_2 (4–5 ml, 10 min) followed by filtration. The filtrate was concentrated *in vacuo*, and the by-products were removed by washing the peptide fraction with dry ether. The linear peptide purity was characterized by electrospray ionization mass spectrometry (Micromass Platform II spectrometer) and analytical HPLC. The linear precursors were cyclized without purification. The peptides (0.3 mmol) were dissolved in DMF (5 ml) added quickly from a syringe to a well-stirred DMF solution (300 ml) containing 1.5 equiv of HBTU and 10 equiv of NEt_3. The reaction mixture was stirred for 30 min at room temperature. The solvent was removed *in vacuo* to leave an oil, which was dissolved in EtOAc (80 ml) and washed successively with 0.1 M citric acid, saturated aq. NaCl, saturated aq. $KHCO_3$, saturated aq. NaCl and water. The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo* to give the crude cyclic peptide. In the case of the compound **3**, the residue was treated with TFA to deprotect tyrosine, threonine and aspartic acid side chains. The compounds **1** and **3** were purified by semi-preparative reversed-phase C_{18} HPLC on a Kromasil column (5 μm, 7.8 × 250 mm, detection at 220 nm); **1** (55 mg; yield: 27%), **3** (33 mg; yield: 31%).

Oxidation of 1

Compound **1** (10 mg) was dissolved in 500 μl of DMSO, then 300 μl of H_2O_2 (35% in H_2O) were added drop by drop and the solution was stirred for 30 min at room temperature. The final product was directly purified on Kromasil C_{18} (250 × 7.8 mm) semi-preparative HPLC to give **2** (8.2 mg, yield: 80%).

Each peptide identity was further confirmed by mass spectral analysis (LSIMS) and the expected MH^+ molecular ions were observed for all the peptides. The cyclic peptides were determined by analytical reversed-phase C_{18} HPLC to be >98% pure.

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